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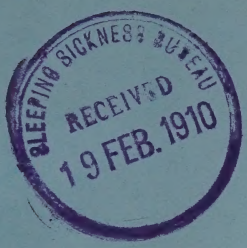

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By LEONARD ROGERS, M.D., M.R.C.P., I.M.S.

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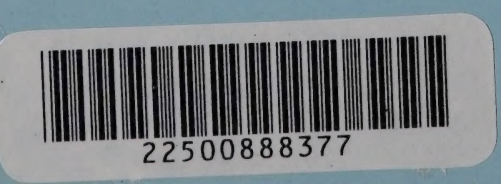

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On the Development of Flagellated Organisms (Trypanosomes) from the Spleen Protozoic Parasites of Cachexial Fevers and Kala-Azar.

By

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(With Plate 25.)

The small oval parasites, known under the name of Leishman-Donovan bodies (although they appear to have been first found by D. D. Cunningham in Dehli boil) were described last year as occurring in the enlarged spleens of patients dying of chronic fever with marked cachexia by Leishman, who considered them to be degenerate trypanosomes, because he found somewhat similar bodies form with a large and a small chromatine mass in the spleens of rats which had died forty-eight hours before of trypanosomiasis due to the organisms of tsetse fly disease. Donovan, working in Madras, found similar bodies in blood obtained fresh from patients suffering from this fever, thus proving that those seen by Leishman were not degenerate trypanosomes, and Laveran, after examining Donovan's specimens, came to the conclusion that the parasite was a piroplasma. Ross, Nuttall, and Manson have all dissented from this view, and regard the organism as probably belonging to a new genus. Christophers suggests that it is a microsporidium.[1]

I have elsewhere shown that the parasite is to be found in

[1] Professor Ray Lankester, in the 'Quarterly Review,' July, 1904, expresses the view that Schaudinn's recently published researches, "On the Trypanosomes of the Blood of the Stone Owl," render it probable that Leishman's corpuscles, as well as those of Delhi sore, are stages in the life-history of a Trypanosoma.

the great majority of cachexial fevers with enlarged spleens occurring so commonly in Calcutta, and also in still larger numbers in all cases of active kala-azar, which, as I maintained in 1897, is nothing but a severe form of the disease hitherto known as "malarial cachexia," but for which I have suggested the more appropriate one of "cachexial fever" until further advances in our knowledge of the new parasite enabled a better one to be decided on. In the course of my recent investigations I tried various methods of studying the parasites outside the body, and eventually found one by which they could be kept alive for some days, during which they multiplied greatly, and in some instances developed new forms of considerable interest. The method by means of which these results have been obtained is an extremely simple one. The blood obtained by spleen puncture was immediately ejected into small sterile test-tubes containing a little sodium citrate to prevent the blood from coagulating, and these were then incubated at varying temperatures, portions of the culture being removed with a platinum loop from time to time for examination with the microscope. At blood heat I found the spleen parasites rapidly underwent degenerative changes, and after twenty-four hours most of them had disappeared and the remainder stained badly. As the presence of a macro- and a micro-nucleus in the spleen parasites pointed to their possible relationship with the flagellated class of protozoa, and it is known that trypanosomes live much longer out of the body at low temperatures than at blood heat, I next tried incubating the culture tubes in a cold incubator at 27° C., ice being used, as the laboratory temperature was several degrees above that point. At this temperature I found that the parasites lived for some days, retaining fully their staining properties. Further, in favourable cases, in which a large number of parasites were present in the blood when first obtained (which is only the case in about one fifth of those met with in Calcutta), it was soon evident that they were undergoing division and increasing very materially in numbers, for, instead of two or three in a field of an immer-

sion lens, as in the original specimens, as many as fifty or more were sometimes seen in the same area in those from the cultures. Moreover, divisional forms, which are rare in fresh spleen blood, appeared in very large numbers in the cultures after from one to three days, thus allowing the modes of division to be much more easily studied.

Divisional Forms without Development.

The divisional forms, which occur in great numbers in cultures at 27° C., are of two kinds. The first is a simple subdivision of the small oval parasites into two, both the macro- and the micro-nucleus first dividing, and then the body of the cell splitting into two, the cleavage beginning at one end, so that just before they separate they remain attached only by the other poles. This mode of division is illustrated in line I of the plate, figs. 1 to 4. These forms can be found in small numbers by long search in films of blood obtained by spleen puncture when numerous parasites are present, but they form only a very small proportion of the total number of organisms seen. On the other hand, in cultures they are present in very much larger numbers, several in various stages being often seen in a single field of the microscope.

The second mode of division is a multiple one, as shown in line I, figs. 5 to 8. The macro- and micro-nucleus divides a number of times, as in fig. 6, instead of only once, the outline of the cell becoming less definite, until eventually the appearance shown in fig. 7 is reached, in which a number of very small nuclei arranged in pairs of a small and a large kind enclosed in a zoogloœa-like material is seen. Next these enlarge gradually, and each pair becomes surrounded by a faint capsule, which becomes more and more distinct with the growth of each young form, until the characteristic groups of the oval bi-nucleated, fully-grown spleen parasites result, as shown in fig. 8 of line II of the plate, which are not very rarely seen in good specimens of spleen puncture blood.

Fig. 8 of line I shows a nearly full-sized group. All stages of these multiple divisional forms occur in large numbers in favourable cultures at 27° C., every stage being sometimes seen in a single field of the microscope. They are found most abundantly in a slimy material, which appears in the tubes after a day or two, and which stains rather like fibrin, but contains very few red corpuscles. This mode of division also takes place within the spleen during life, probably accounting for the greater number of the parasites, and the different stages can be seen in smears made from the organ shortly after death. The smallest multiple form is, however, very rarely seen in films of blood obtained by spleen puncture, probably because the cells, distended by a number of the larger forms, are more readily ruptured by the suction action of the syringe than are those containing the smaller forms. The formation of these multiple young forms in a zoogloœa-like material derived apparently from the protoplasm of the dividing parasite itself, and occurring in culture-tubes in which the blood-corpuscles have broken down, clearly proves that the parasites are not growing in the red corpuscles, and thus renders Laveran's contention that the parasites are piroplasma untenable.

At a temperature of 27° C. only the above-described forms were seen in large numbers. Noye's blood-agar culture medium was also tried without success. On next reducing the temperature of the cold incubator down to about 22° C. and making further cultures in a new series of cases of citrated spleen blood, further and more important changes were soon found.

Developmental Forms.

The first thing noticed was an enlargement of the small oval spleen parasites, affecting especially the macro-nucleus and the protoplasm of the cell, the micro-nucleus remaining unchanged. Then one day a culture of only twenty-four hours' growth, the fully developed flagellated forms shown

in figs. 8 to 12 of line XI of the plate, were suddenly encountered, together with the intermediate forms shown in the first seven figures of the same line. Since that time a number of cultures have been made and further intermediate forms have been met with, but in these it has taken three or four days before large flagellated forms were found, and the fully elongated trypanosoma-like forms of case 37 have not again been seen so perfectly. What the conditions were which favoured the full development in so short a time in that case I cannot say. The case was a more acute one than is often seen in Calcutta, but a second lot of spleen blood obtained a few days later failed to develop in the same way, so there must have been some other factor present. As in all my other successful cultures the steady development of the parasites day by day could readily be traced, it will be best to describe these changes in the order of their development. For the purpose of illustrating the progress of the evolution the forms seen each day in two cases have been drawn in the plate, each line representing one day's appearances.

Stage of Development after Twenty-four Hours. —At the end of one day at 22° C. an examination of the citrated blood shows the forms figured in lines III and VII of the plate, while lines II and VI show those seen in the spleen blood of the same cases before incubation. It will be seen from line III that at the end of one day the organisms have already increased considerably in size, while the macro-nucleus is also larger, this being a striking feature. On the other hand, the micro-nucleus has not altered, but still remains small and rod shaped. The forms shown in line VII also show that the macro-nucleus, in addition to being larger, is beginning to present a granular appearance, while it does not stain so darkly as in the original spleen parasites. Further, the protoplasm of the cell is also increasing in amount and now take on a bluish staining, and has a very finely granular appearance. These are the only changes met with as a rule on the first day.

Stage of Development after Forty-eight Hours.—By the end of the second day much more marked changes are met with, the principal forms of which are shown in lines IV and VIII of the Plate. In the first place there is a still further and very marked increase in the size of the organisms still affecting especially the macronucleus and the protoplasm, as in figs. 5 and 7 of line IV. Secondly, and of much greater interest, is the appearance of double forms, such as are not met with on the first day. These show every degree from apposition at one point of their circumference of two of the large oval forms, through closer degrees of contact up to nearly complete fusion of the two cells. At first I took these stages for a method of division, but as a further study showed that the latter developments into elongated and flagellated forms always takes place in pairs or rarely threes, I have come to the conclusion that these early double forms are really a kind of conjugation, such as is known to occur in other protozoa preparatory to the evolution of new stages in their life history. In favour of this view there is also the fact that the pairs of large oval organisms during the second and third days are found to be in contact with each other in very varying positions, and to present no regularity in this feature, as is the case with the small spleen forms undergoing fission shown in figs. 1 to 4 of line I of the Plate. Thus, while figs. 4 and 6 of line IV show contact of the sides of the oval bodies, figs. 5 and 7 of line V show apposition of the end of one to the side of the other, and similar variations are shown in the figures of line VIII.

In addition to the forms showing mere apposition, others show more or less complete degrees of fusion of two oval forms, as in figs. 1, 2, and 8 of line IV, the two macro- and micronuclei being each distinctly seen. Further, even on the second day, forms approximating to the next stage in the development of the organism may be found—namely, an elongation of the conjugating forms, as shown in figs. 1, 6, and 7 of line VIII,—but as a rule these do not appear in any numbers until the third day.

Stages of Development after Seventy-two Hours. —The third day is characterised by the elongation of the conjugating pairs of organisms, and the first appearance of flagellated forms, although sometimes the latter may not be found until the fourth day. The commonest appearance of these pyriform bodies is that shown in fig. 1 of line V, in which the macronuclei are seen in the thick ends of the organisms, while the micronuclei have passed to the thinner ends from which the flagella will eventually arise. In Case 58, from which the figures of lines II to V have been drawn, the culture-tube was unfortunately left out of the cold incubator for half an hour owing to an interruption in my work, and no further development occurred although the temperature of the laboratory was only 28° C. at the time ; so sensitive are the partially-developed forms to a rise of the thermometer. In Case 47 some early flagellated forms were found on the third day, as shown in figs. 4, 5, and 6 of line IX. In these only a single flagellum has yet developed although two of the forms are distinctly double ones, while some which appear to be single are really double ones lying on one side, for intermediate appearances showing the double nuclei partially obscuring each other in this manner have been met with. The remaining forms shown in line IX have all reached the elongated stage although still without flagella.

Stage of Development after Ninety-six Hours.— In the figures of line X are shown some of the flagellated forms found on the fourth day in Case 47, in addition to which there were much more numerous double pyriform organisms without flagella, for only a very small percentage of the conjugating forms eventually reach the flagellated stage under the artificial conditions of the cultures, which must be very far from being as favourable to the development of the organism as the natural conditions in which it takes place, whatever they may be. Nevertheless, the elongated flagellated forms have now been found in eight different cases, including two of kala-azar from Assam. In fig. 3 of line X the two flagellated bodies have apparently just separated.

Very occasionally groups of three instead of two organisms are found both in the early conjugating stage and in the later elongated and flagellated forms, as shown in fig. 2 of line X.

The Trypanosome-like Stage of Development.—From the forms so far described all that could safely be said is that flagellated organisms with an elongated body and micronucleus at the flagellated end have been obtained, but it could hardly be called a definite trypanosome. However, the forms shown in line XI of the plate go far to support the view that the organism is really a trypanosome, these having been found in a one day culture of Case 37, in which the conditions must have been in some unknown way much more favourable to the development of the organism than in the other cases. The forms shown in figs. 8 to 12 of line XI are precisely like the flagellated forms described above, except that they have elongated out to a much greater degree, so as to very closely resemble trypanosomes in everything except the absence of an undulating membrane, but this is known to be absent in very young trypanosomes, so that it would be expected to be the last feature to be developed in the growth of the organism from the plasmodial spleen form. The double forms shown in figs. 8, 10, and 12 of line XI are of great interest as an indication that these trypanosome-like forms have also developed in pairs, as in the more pyriform flagellated forms shown in line X. Further, fig. 9 of line XI was one of two precisely similar forms lying close together as if they had just separated, as in fig. 3 of line X. Moreover, the figs. 2, 3, and 4 of line XI are precisely similar in nature to the early stages of Cases 47 and 58 already described, from which they only differ in the greater elongation of 3 and 4. A possible explanation of the more typically trypanosome-like appearance of the flagellated forms of Case 37 is that as they developed within twenty-four hours, instead of only after three or four days as in the other cases, they must have found the blood in which they were growing much less altered than it is after several days' incubation in a test-

tube, and consequently, the conditions being less unnatural, their development has more nearly approached the typical form of trypanosomes.

Amœboid Forms.—The small flagellated forms represented in figs. 1, 5, 6, and 7 of line XI are also of great interest, for they correspond very closely with the forms of Trypanosoma Brucii described by Rose Bradford and Plimmer in the 'Quarterly Journal of Microscopical Science' of February, 1902, as "amœboid" stages, and found by them in the lungs of animals affected by tsetse fly disease. The origin of the flagella from the micronuclei is well seen in figs. 6 and 7 of this series, which I have only found in this case, although that shown in fig. 5 has been met with in others as well. As these very delicate organisms do not appear to form part of the regular cycle of development of the trypanosome stage from the spleen parasites, it appears to me to be possible that they may be a portion of the life-history of the parasite which is well fitted to live in the circulation, and which might conceivably be carried from one patient to another by the bites of flies and mosquitoes without undergoing any development within the insects, just as I showed in a previous paper the trypanosoma of surra may be carried from one animal to another by the bites of horse flies in a purely mechanical manner, an observation which has since been confirmed both in South America and in the Philippine Islands. In this connection it is worth while recalling the fact that when Indian cattle are inoculated with the surra trypanosoma they suffer from only a mild chronic form of the disease, and the trypanosomes are only found in their blood for a few days after a definite incubation period. Nevertheless, they every now and then get attacks of fever for many months afterwards (very like the repeated attacks in cachexial fever and kala-azar), but trypanosoma can no longer be found in their blood at such times by ordinary microscopical examination. Nevertheless, I found that if a little of their blood, taken during one of these periodical attacks of fever, is inoculated into a susceptible animal they

readily contract a fatal form of surra with innumerable trypanosoma in their blood. It is possible that a small amœboid stage of the parasite is the infective agent in such cases, and that in a similar way the infection of cachexial fever may be due to some such form carried from one person to another by the bites of flies and mosquitoes. The fact, which I pointed out some years ago in the case of kala-azar, that the infection is very largely a house one and always extremely localised (the movement of healthy people from an infected line to a new site half a mile or so away which I recommended having proved successful in preventing the spread of the disease), is in favour of such a mode of infection.

It is also worthy of note that the plasmodial form of T. Brucii described by Rose Bradford and Plimmer very closely resembles the parasites found in the spleen of these chronic fevers in man and the small multiple forms in my tubes; so that in this disease I have now obtained in cultures the plasmodial, amœboid, and flagellated forms found by those authors in a variety of animals after long study of the disease produced by the T. Brucii; a fact which can leave but little room for doubt that the human parasite belongs to the trypanosomes. Successful inoculation experiments are still wanting to prove this, all the animals I have tested—including tank fish (which are commonly infected with a sluggish, much curved, double S-shaped trypanosome) having proved insusceptible even when injected with cultures containing the large flagellated form of the parasite; but further work based on the knowledge of the true nature of the organism now available should lead in time to further elucidation of a disease which is certainly second to none in the frequency and seriousness of the illness it produces in many parts of India, and also appears to be widely distributed in other countries.

EXPLANATION OF PLATE 25,

Illustrating Mr. Leonard Rogers' paper "On the Development of Flagellated Organisms (Trypanosomes) from the Spleen Protozoic Parasites of Cachexial Fever and Kala-Azar."

All the drawings in this plate were made from the actual specimens by the Medical College artist, Behari Lal Das, as seen under a $\frac{1}{12}$ lens and a No. 4 ocular, the magnification being 925 diameters. The preparations were all stained with Romanosky's stain, used by Leishman's method.

Line I, Figures 1 to 4, show the simple method of division of the spleen parasites, and 5 to 8 the multiple form of division.

Line II shows the organisms present in a film made from freshly obtained spleen blood, Figure 8 representing a group of young parasites.

Line III shows the forms found after one day's incubation of the same blood, the parasites showing only enlargement.

Line IV shows the same after two days, both single large oval forms and conjugating ones being represented.

Line V shows the same at the end of three days, both conjugating forms and elongated pairs being present.

Lines VI to IX show similar development day by day of Case 47, the early flagellated forms being seen in Figures 4 to 6 of Line IX.

Line X shows the large flagellated pairs, with the flagella arising from the ends containing the micronuclei.

Line XI shows all stages of the development from a one day culture of Case 37. Figures 8 to 12 represent the fully developed long trypanosome-forms with macro- and micro-nucleus, three of which still show the double form of the typical development. Figures 1, 5, 6, and 7 show the small flagellated amœboid forms resembling those found by Rose, Bradford, and Plimmer in Trypanosoma Brucii.

Huth. sc et imp.